UNITED NATIONS

SECRETARIAT

ST

Distr.
GENERAL
ST/SOA/SER.J/27
30 December 1976
Original: ENGLISH

SCIENTIFIC RESEARCH ON PAPAVER SPECIES AS SOURCES OF CODEINE, MORPHINE AND THEBAINE [1]

No. 27

HIGH PRESSURE LIQUID CHROMATOGRAPHIC SEPARATION AND QUANTITATION OF THEBAINE FROM PAPAVER BRACTEATUM [2,3]

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1/ This ST/SOA/SER.J/.. series of documents was previously entitled "Scientific research on Papaver bracteatum".

2/ In the report of the Second Working Group on P. bracteatum (document ST/SOA/SER.J/2), it was recommended that methods for the determination of thebaine in P. bracteatum should be compared and evaluated; to achieve this, it was decided that participants in the Working Group would forward their methods for publication in this series so that these could be tried out in the various laboratories.

3/ This research was supported by S.B. Penick and Company, Orange, New Jersey.

4/ This paper was submitted by Dr. Bernard B. Brown, Vice President of Research, S.B. Penick and Company.

General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.

GE. 77-1513

Gas chromatography has been used routinely to separate and quantitate the thebaine in Papaver bracteatum [5-10]. However, none of these GC procedures has established that the thebaine peak represents pure thebaine. For this reason, a total alkaloid extract of P. bracteatum was injected on a GC column [11] and the compounds represented by the two resulting peaks were collected. GC operating conditions were: injector and detector temperature, 285°C; oven temperature, 270°C; flow rate of carrier gas (helium), 48 ml/min.; hydrogen pressure, 17.5 p.s.i.g. The two fractions obtained were each dissolved in chloroform and the samples were applied to a precoated silica gel GF_{254} thin-layer plate (0.25 mm) [12]. After developing with toluene-acetone-ethanol-conc. ammonium hydroxide (20:20:3:1) [13], the chromatogram was examined under both long and short wave UV light and sprayed with sulfuric acid [14] followed by charring with heat. Thebaine was apparently decomposed by the high temperature used

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5/ P. Cheng and N.J. Doorenbos, United Nations document ST/SOA/SER.J/5 (1973).

6/ F.J.E.M. Kuppers, R.J.J. Ch. Lousberg, C.A.L. Bercht and C.A. Salemink, United Nations document ST/SOA/SER.J/8 (1974).

7/ P.G. Vincent and W.A. Gentner, United Nations document ST/SOA/SER.J/9 (1974).

8/ M.J. Duffy, P.A. Ackert and C.F. Hiskey, United Nations document ST/SOA/SER.J/18 (1975).

9/ D. Furmanec, J. Chromatogr. 89, 76 (1974).

10/ C.B. Coffman, C.E. Bare, and W.A. Gentner, Bull. Narcot., 27-3, 41 (1975).

11/ A Perkin-Elmer Model 881 gas chromatograph equipped with a hydrogen flame ionization detector and a Seargent SR recorder were used in this study. A spiral glass chromatographic column (6 ft. long, 1/4 in. O.D.) was packed with 2.5% OV-17 on 100-120 mesh Gas-Chrom Q (Applied Science Laboratories Inc. State College, Pennsylvania).

12/ E. Merck, Darmstadt, Federal Republic of Germany.

13/ E. Stahl, H. Jork, E. Dumont, H. Bohrmann and H. Volmann, Arzneimittel. Forsch., 19, 194 (1969).

14/ Concentrated sulfuric acid dissolved in methanol (70% v/v).

during gas chromatography, since no spot corresponding to thebaine could be observed. The two collected fractions were qualitatively identical, containing at least four compounds but were quantitatively different. It was also observed that the ratios of one compound to another in each fraction was dependent on the column temperature employed. Since thebaine is decomposed under the conditions used in GC analysis, it is impossible to determine whether the thebaine peak represents pure thebaine or a mixture of phyto-constituents and/or their decomposition products. For this reason, a high pressure liquid chromatographic procedure employing milder conditions was developed.

A number of high pressure liquid chromatographic separations of thebaine and other opium alkaloids have been previously reported [15-22]. However, most of these methods require either pre-treatment of the extract to remove polar impurities (e.g., ion-exchange chromatography) or gradient elution equipment, and/or they do not produce base line separation of thebaine

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15/ D.W. Smith, T.H. Beasley, Sr., R.L. Charles, and H.W. Ziegler, J. pharm. Sci., 62, 1691 (1973).

16/ I. Jane, J. Chromatogr., 111, 227 (1975).

17/ J.D. Wittwer, Jr., J.forensic Sci., 18, 138 (1973).

18/ T.H. Beasley, D.W. Smith, H.W. Ziegler and R.L. Charles, J. Ass. offic. anal. Chem., 57, 85 (1974).

19/ C-Y. Wu, S. Siggia, T. Robinson and R.D. Waskiewicz, Anal. Chim. Acta, 63, 393 (1973).

20/ J.H. Knox and J. Jurand, J. Chromatogr., 82, 398 (1973).

21/ I. Jane and J.F. Taylor, J. Chromatogr., 109, 37 (1975).

22/ P.J. Twitchett, J. Chromatogr., 104, 205 (1975).

from other P. bracteatum constituents. A rapid, single column separation which produces base line separation of thebaine, but which does not require prior removal of polar impurities or gradient elution equipment was sought.

Preparation of plant extracts for chromatography

Dried, 40-mesh P. bracteatum (1.0 g) was transferred to a 125 ml Erlenmeyer flask and 5% aqueous acetic acid (50 ml) added. The flask was shaken for one hour on a rotary shaker, the mixture was filtered through a Büchner funnel and the marc was washed with 5% aqueous acetic acid (10 ml). Following alkalinization of the filtrate with concentrated ammonium hydroxide (6 ml), it was extracted three times with chloroform (50 ml each extraction). The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered, and the sodium sulfate was washed with chloroform (10-15 ml). The combined extracts were evaporated to dryness in vacuo, yielding a residue which was suitable for direct application to a chromatographic column.

High pressure liquid chromatography [23/]

The residue from the chloroform extract was dissolved in an accurately measured volume of methanol-water (8:2), containing 0.3% ammonium carbonate and an aliquot was injected onto a μBondapak C_{18} column [24/] (1 ft. long, 3/8 in. O.D.). Operating conditions were: ambient temperature; flow rate of eluting solvent (methanol-water containing 0.3% ammonium carbonate, 8:2), 1 ml/min.; wavelength of UV detector, 285 nm. Under these conditions, the following retention times were obtained: thebaine, 10.2 min.; isothebaine, 6.0 min.; and orientalidine, 6.0 min. Although orientalidine and isothebaine are not resolved, a sharp, symmetrical peak with base line separation was obtained for thebaine, and the entire separation and quantitation procedure required only 12 minutes.

23/ These studies were conducted using a Waters Associates Model 6000 liquid chromatograph equipped with a Beckman 25 variable wavelength UV spectrophotometer and recorder.

24/ Waters Associates Inc., Milford, Massachusetts.

Although μBondapak C_{18} consists of a C_{18} alkane chemically bonded to silica *via* a hydrolytically stable ether linkage, repeated use of these columns appeared to give rise to a small number of active sites on the silica. It was therefore necessary to saturate these active sites by making a daily injection of 20 μg thebaine standard. In addition, injections of 5 μg thebaine standard were made until successive injections gave thebaine peak areas within two percent of each other or better [25].

In order to determine whether the thebaine peak represents only pure thebaine, twelve repeated injections of *P. bracteatum* straw extracts were made and the thebaine fractions were collected, combined, and evaporated to dryness *in vacuo* to yield approximately 150 μg of residue. This residue was dissolved in chloroform and the total sample was applied to a pre-coated silica gel GF_{254} thin-layer plate (0.25 mm) [12]. The chromatogram was developed a distance of 15 cm. from the point of sample application using toluene-acetone-ethanol-conc. ammonium hydroxide (20:20:3:1) [13]. The developed chromatogram was examined under both long and short wave UV light and then sprayed with sulfuric acid [14] and charred by heating. Only a single spot was observed, corresponding to thebaine (R_f 0.34) [26]. In a second experiment, six repeated injections of *P. bracteatum* straw extracts were made and the thebaine fractions were collected, combined, and evaporated to dryness in vacuo to yield 30 μg of residue. The UV spectrum of this residue dissolved in methanol was superimposable with that of thebaine standard. The purity of the collected thebaine sample was confirmed by thin-layer chromatography.

25/ Usually two injections of 5 μg each are sufficient.

26/ In this chromatographic system, isothebaine and orientalidine had R_f values of 0.40 and 0.52 respectively. Isothebaine and orientalidine were generously provided by Professor E. Brochmann-Hanssen, University of California, San Francisco.

Two standard Beer's law plots were obtained by injecting different concentrations [27] of standard thebaine on the column in quadruplicate and measuring the thebaine peak areas by the triangulation method. In both cases linear plots were obtained with reliabilities of 0.99943 and 0.99928.

Thebaine can therefore be rapidly separated from a P. bracteatum alkaloid extract and quantitated using a single high pressure liquid chromatographic system. This procedure also permits the collection of the thebaine peak and the analysis of this thebaine for purity using other techniques (TLC, UV, mass spectrometry).

27/ The concentrations used were: 0.2, 0.4, 1.0, 1.5 and 2.0 µg (0.25 absorbance units full scale on UV recorder) and 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 µg (0.5 absorbance units full scale on UV recorder).